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PRINCIPAL INVESTIGATOR: Thomas A. Gardner, M.D.

CONTRACTING ORGANIZATION: Indiana University
Bloomington, IN 46202-5167

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Thomas A. Gardner, M.D.

Email: thagardn@iupui.edu

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14. ABSTRACT

This award was to support the infrastructure and development of a Phase I clinical trial for men with metastatic prostate cancer. The Phase I trial under development employs a prostate restricted replicative adenovirus (PRRA) with excellent preclinical performance in vitro and in vivo in relevant animal models of human prostate cancer. Several components of the statement of work for this award have been completed. The key component of this trial the PRRA, AdIU1, required a slight modification before proceeding with implementation of the trial as submitted. Reconfirmation of the activity of the modified AdIU1 was required prior to moving forward with the clinical trial. A majority of the funding from this award remains in the CTDA account and a no cost extension has been requested to allow continued development of the trail in order to apply for a 2007 DOD Clinical Trial Award to initiate a trial in 2008. Currently with the clinical trial team in place and modified AdIU1 verified, the regulatory applications on hold can be filed.

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Final Report

Men with advanced and metastatic prostate cancer continue to have limited treatment options. Hormonal manipulation, radiation therapy and recently chemotherapy have delayed onset of symptoms and have helped palliated those men with symptoms but novel treatment options are required to advance the care of these men. Gene therapy continues to demonstrate pre-clinical and clinical efficacy in several forms.[1, 2] This award was to facilitate the development of the infrastructure of a Phase I clinical trial for men with metastatic prostate cancer. The Phase I trial under development employs a prostate restricted replicative adenovirus (PRRA) with excellent preclinical performance in vitro and in vivo in relevant animal models of human prostate cancer. [3, 4] Several components of the statement of work for this award have been completed. The key component of this trial the PRRA, AdIU1, required a slight modification before proceeding with implementation of the trial as submitted. The initial form of ADIU1 approached the packaging capacity of the adenoviral vector and decreased the viral yield in larger scale preparation, and may have complicated clinical grade viral production. The modified ADIU1 with a decreased packaging size demonstrate viral yields similar to wild type adenovirus. Reconfirmation of the activity of the modified AdIU1 was required prior to moving forward with the clinical trial. (Appendix 2) A majority of the funding from this award remains in the CTDA account and a no cost extension has been requested to allow continued development of the trail in order to apply for a 2007 DOD Clinical Trial Award to initiate a trial in 2008. Currently with the clinical trial team in place and modified AdIU1 verified, the regulatory applications on hold can be filed. With the completion of the manuscript in Appendix 1 submitted to *Molecular Therapy* an application to Center for Biologics Evaluation and Research (CBER), Office of Cellular, Tissue and Gene Therapies (OCTGT), will be submitted for review at the next meeting.

Key Award Accomplishments

1. Completion of the Study Team with monthly meeting to discuss trial development
   Urologist: Thomas A. Gardner, M.D.
   Oncologist: Noah Hanh, M.D.
   Radiation Oncologist: Song-chu Ko, M.D./Ph.D.
   Research Coordinator: Rhoda Loman

2. Confirmation of simplified product development (Appendix I)

References:

1. Freytag, S.O., et al., *Five-year Follow-up of Trial of Replication-competent Adenovirus-mediated Suicide Gene Therapy for Treatment of Prostate Cancer.* Mol Ther, 2007. **15**(3): p. 636-42.
2. Desai, P., et al., *Future innovations in treating advanced prostate cancer.* Urol Clin North Am, 2006. **33**(2): p. 247-72, viii.
3. Lee, S.J., et al., *Novel prostate-specific promoter derived from PSA and PSMA enhancers.* Mol Ther, 2002. **6**(3): p. 415-21.
4. Li, X., et al., *Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer.* Cancer Res, 2005. **65**(5): p. 1941-51.

Appendix I

Tumor-specific suicide gene therapy for prostate cancer using m6 promoter-driven herpes simplex virus thymidine kinase and ganciclovir

Miwon Ahn,[1,4] Xiong Li,[1,4] Sang-Jin Lee,[1,4] Juan Antonio Jiménez,[1,2,4] Yan-Ping Zhang,[1,4] Kyung-Hee Bae,[1,4] Yousef Mohammadi[3], Chinghai Kao,[1,2,4] and Thomas A. Gardner[1,2,4]

Departments of [1]Urology, [2]Microbiology and Immunology, [3]Biology and [4]Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana.

Corresponding Author:
Thomas A. Gardner, MD
Indiana University School of Medicine
Department of Urology
535 Barnhill Drive
Suite 420
Indianapolis, IN 46202
Phone: 317-278-3434; Fax: 317-274-0174; E-mail: thagardn@iupui.edu

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Abstract

Enzyme pro-drug suicide gene therapy has been hindered by inefficient gene delivery and transduction. To further explore the potential of this approach, we have developed AdIU1, a prostate-restricted replicative adenovirus (PRRA) armed with the herpes simplex virus thymidine kinase (HSV-TK). This suicide gene (*HSV-TK*) and pro-drug (ganciclovir (GCV)) combination has been extensively explored in both preclinical and clinical studies. In our previous Ad-OC-TK/ACV phase I clinical trial, we demonstrated both safety and proof of principle with a tissue-specific promoter-based TK/pro-drug therapy using replication defective adenovirus in treating prostate cancer metastasis. In this study, we aimed to inhibit the growth of androgen-independent (AI), PSA/PSMA-positive prostate cancer cells by AdIU1. *In vitro*, the growth of an androgen-independent PSA/PSMA-expressing prostate cancer cell line, CWR22rv, was significantly inhibited by treatment with AdIU1 plus GCV (10 μg/ml), compared to AdIU1 treatment alone. On the other hand, AdE4PSESE1a (a PRRA lacking HSV-TK) with and without GCV (10 μg/ml) treatment demonstrated similar *in vitro* cytotoxicity as AdIU1 alone. *In vitro* cytotoxicity was observed following treatment with AdIU1 plus GCV only in PSA/PSMA-positive CWR22rv and C 4-2 cells, but not in the PSA/PSMA-negative cell line, DU-145. *In vivo* assessment of AdIU1 plus GCV treatment revealed a stronger therapeutic effect against CWR22rv tumors in nude mice than treatment with AdIU1 alone, AdE4PSESE1a or AdE4PSESE1a plus GCV. In summary, we have developed a novel therapeutic strategy for the treatment of AI prostate cancer.

INTRODUCTION

Prostate cancer is still the leading cancer diagnosis in men. The incidence of prostate cancer is age-dependent and has steadily increased over the last several decades [1]. Localized prostate cancer can be managed effectively with surgery or radiation, while advanced and metastatic disease eventually progresses to an androgen-independent (AI) state with limited treatment options. The aging population of men with an increasing prostate cancer incidence combined with an absence of successful therapies for advanced disease require the development of novel therapies.

Suicide gene therapy is a novel approach to increase drug selectivity towards cancer cells. Tumor-specific suicide gene therapy using a tissue-specific promoter is a rational treatment strategy for prostate cancer [2-6]. Herpes simplex virus *thymidine kinase* (*HSV-TK*)-based suicide gene therapy has been used to target prostate cancer for over a decade [7-10]. The pro-drug, ganciclovir (GCV) is phosphorylated by HSV-TK to its monophosphate form, which is rapidly converted to di- and triphosphate forms by cellular kinases, the latter of which is toxic to cells. The GCV- triphosphate is incorporated into DNA during cell division, causing single-strand DNA breaks and inhibition of DNA polymerase [10-12] and causes DNA chain termination, which leads to programmed cell death.

Tumor-specific oncolytic adenoviruses have been effective and safe treatment options for patients with metastatic disease. Several studies have demonstrated the importance of tissue-specific vectors, revealing systemic toxicity with the administration of high doses of nonspecific vectors. Through the use of prostate-specific promoters and enhancers, the expression of a therapeutic gene or adenoviral replication can be limited to cells that contain the appropriate activators and transcription factors. Currently, *kallikrein 2*, *PSA*, *rat probasin*, and *osteocalcin (OC)* are each under extensive investigation as regulators of prostate restricted replication adenovirus [13-18]. We have demonstrated that both the prostate-specific antigen (PSA) and osteocalcin (OC) promoters could transcriptionally regulate the HSV-TK gene in a prostate-specific manner *in vitro* and in *vivo* [19]. This tissue-specific HSV-TK production combined with pro-drug administration inhibited the growth of Al-PSA-producing cells *in vitro*, in animal models of human prostate cancer and in patients with prostate cancer enrolled in a Phase I clinical trial of OC promoter-based HSV-TK gene therapy [19, 20]. Others have demonstrated similar *in vitro* and *in vivo* efficacy as well as safe administration to men with locally advanced and metastatic prostate cancer [21, 22]. More recently, Freytag *et al.* demonstrated the safety and efficacy of a conditionally replicating, non-tissue-specific adenovirus containing the suicide genes *TK* and *CD* when combined with external beam radio therapy [23-25]. Although Ad5-CD/TKrep/prodrugs/radiation therapy demonstrated a promising result in locally recurrent prostate cancer, the expression of the CD/TK fusion gene under the control of strong universal CMV promoter also showed lack of tissue specificity, which severely impairs the safety of this virus. Besides, Ad5-CD/TKrep is based on the E1B 55k deleted virus, del520/ONXY-015. Recent

studies indicated that the replication of del520 virus is p53-independent. [26-29]. In addition, del520 exhibits substantial replication in certain normal cells. These complications will potentially limit the use of Ad-CD/TKrep. Nevertheless, ad5-CD/TKrep warrants further development as suicide gene therapy.

We recently developed a prostate-specific chimeric enhancer, *PSES*, by combining enhancers from *PSA* and *PSMA* genes. Prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) are prostate-specific biomarkers expressed by the majority of prostate tumors and non-cancerous epithelium. The main prostate-specific enhancer activity of the PSA enhancer core is located in a 189 bp region called AREc3, and the main prostate-specific enhancer activity of the PSMA enhancer core is located in a 331 bp region called PSME(del2). The combination of these two regulatory elements, AREc3 and PSME(del2), called *PSES*, showed high activity specific to PSA/PSMA-positive prostate cancer cells, regardless of androgen status [30]. This *PSES* promoter has been used to control the replication of a PRRA, which demonstrated prostate-specific replication and therapeutic efficacy both *in vitro* and *in vivo* [31]. We also made a shorter form by deleting L2 and L5 region in AREc3 and replacing the 90bp proximal region of PSME with simple AP-3 binding site of PSES, called m6. For this study, we developed a novel *HSV-TK*-armed tissue-specific replicative adenovirus, AdIU1, using the *PSES* promoter to drive the expression of adenoviral E1a and E4 and the m6 promoter to drive the expression of adenoviral E1b and *HSV-TK*. AdIU1 demonstrated selective cytotoxicity toward androgen-independent (AI) PSA/PSMA-expressing prostate cancer both *in vitro* and *in vivo*.

RESULTS

Construction of a shorter form of *PSES*, m6

We constructed a HSV-TK armed PRRA by replacing the CMV-GFP expression cassette in AdE4PSESE1a [31] with a PSES-HSV-TK expression cassette. However, the virus did not propagate well in 911E4 cells (unpublished data). We believe the problem was the size limitation of the adenovirus. We modified the *PSES* sequence by deleting the non-functioning sequences [30]. Our linker mutation study indicated that L2 and L5 likely did not have a function (Fig. 1A, [30]). Also, the activity in 90 bp upstream region in AREc3 of PSME del2 was mediated by AP-3 (Fig. 1B) [32]. We decided to make a shorter form of PSES, called m6, by deletion of the L2 and L5 sequence of AREc3 and replaceing the 90 bp upstream sequence of PSME del2 with a simple Ap-3 binding sequence Figure 1C demonstrates that m6 retained tissue specific activity in PSA and PSMA positive cells.

Construction of a TK-armed PRRA.

AdIU1 was constructed by replacing the *CMV-GFP* expression cassette in AdE4PSESE1a [31] with a *m6-HSV-TK* expression cassette to extend the therapeutic potential of the PSES-based PRRA (Fig. 2). HER 911E4 cells were transfected with recombinant adenoviral cosmid linearized by Pac I restriction enzyme digestion, and AdIU1 was propagated in HER 911 E4 cells as described in Material and Methods. CsCl-purified AdIU1 was tittered with the Adeno-XTM rapid titer kit. The titer was expressed as infectious units (IFU). To assess the prostate specificity and viral replication efficiency of AdIU1, *in vitro* viral replication assay was performed. PSA/PSMA-positive and negative cells were infected with a dose of AdIU1 different from adenovirus's similar infectivity [31]. AdIU1 replicated as efficiently as AdE4PSESE1a in PSA/PSMA positive C 4-2 and CWR 22rv cells (Table 1). The replication was dimmed in PSA/PSMA- negative PC 3 and DU-145. Demonstrating the fact that AdIU1 replication is tightly controlled by PSES and restricted to PSA/PSMA positive cells.

Western blotting analysis of adenovirus E1a and herpes simplex virus thymidine kinase proteins expression.

AI, PSA/PSMA-positive CWR22rv and C 4-2, as well as AI, PSA/PSMA-negative DU-145 and PC 3 were infected with standardized doses of AdIU1. 48 hrs post AdIU1 infection, cell lysate was collected and Western blot was performed using monoclonal antibodies against Ad5E1a or polyclonal HSV1-TK antiserum. Ad5E1a and HSV1-TK proteins expression were detected in AdIU1 infected, PSA/PSMA positive CWR22rv and C 4-2 cells. On the other hand, the expression of Ad5E1a and HSV1-TK proteins, in AdIU1 infected PSA/PSMA-negative PC 3 and DU-145 cells were low or undetectable

(Fig. 3). This result indicated that PSES and m6 promoter retained it prostate specificity to mediated E1a and HSV-TK expression in AI, PSA/PSMA positive cells.

Selective cell killing activity of AdIU1 plus GCV against AI, PSA/PSMA-positive human prostate cancer cells *in vitro*.

The Pro-drug sensitivity assay *in vitro* was performed. Each cell line, CWR22rv, C 4-2 and DU-145 were seeded in triplicate in 24-well plates at a density of 1×10^5 or 2×10^4 cells/well and were incubated with increasing concentrations of GCV (0 to 100 μg/ml). Cell viability was determined after 5 days using crystal violet assay, and a corresponding IC_{50} dose was determined for each cell line (data not shown). The optimal non-toxic GCV treatment dose was determined to be 10 μg/ml.

To evaluate the selective cytotoxicity of AdIU1 and AdE4PSESE1a viruses, we infected each cell line with wide dose ranges (0.0002 - 2 IFU/cell) of virus, and then treated infected cells with or without GCV (10 μg/ml) (Fig. 4A). The growth of AI, PSA/PSMA-positive human prostate cancer cell, CWR22rv was significantly inhibited by 0.0002 IFU of AdIU1 in the presence of GCV. AdIU1 without GCV had similar killing activity as AdE4PSESE1a either in the presence or absence of GCV. The growth of the AI, AR- and PSA/PSMA-negative cell line, DU-145 was unaffected by either virus with or without GCV. In a time course experiment, CWR22rv and DU-145 cells were seeded in-24 well plates. The 24 wells were divided into 4 groups, no treatment, AdIU1, GCV alone, and AdIU1 plus GCV treatment. The GCV alone group demonstrated limited cytotoxicity. This confirmed that 10 μg/mL GCV treatment was not toxic to either prostate cancer cell lines. The CWR22rv cell line demonstrated cell growth inhibition at day 7 after AdIU1 exposure. The killing activity was significantly enhanced when GCV was administered following AdIU1 infection (Fig. 4B). The DU-145 cell line demonstrated limited cytotoxicity in all four treatment regimens (Fig. 4C).

***In vivo* growth inhibition of CWR22rv xenograft by AdIU1/GCV**

Human prostate CWR22rv xenograft tumors were induced by subcutaneous injection of CWR22rv cells into athymic nude mice. The mice were castrated 3 days after CWR22rv inoculation to test whether AdIU1 or AdE4PSESE1a was able to eliminate AI tumors in a castrated host. After tumor formation, the mice were randomized into 4 treatment groups (AdIU1, AdE4PSESE1a, AdIU1 plus GCV and AdE4PSESE1a plus GCV). The mice were injected intratumorally with AdIU1 or AdE4PSESE1a. Day 0 was the time of virus injection. On day 5, groups receiving GCV treatments were injected with GCV (40 mg/kg body weight) 2 times a day for 10 days. Tumor volumes were measured at the times indicated in Figure 5. AdIU1/GCV effectively caused growth delay of CWR22rv xenografts. Light microscopic observation of H&E-stained tissue sections from tumors injected with AdIU1/GCV showed

substantial treatment effect (Fig. 6). We observed a large amount of fibrosis following combined treatment. Also, we observed that all necrotic tumors stained positive for apoptosis by TUNEL assay (Fig. 7 a, b, c, d). We observed no significant difference in apotosis between both groups at 30 day after injection. Anti-adenovirus type 5 E1a immunohistochemical staining revealed that extensive viral infection existed throughout the AdIU1, AdE4PSESE1a, and AdE4PSESE1a plus GCV treatment group tumors (Fig. 7 e, f, g, h); however, adenovirus staining was absent in the AdIU1 plus GCV treatment group.

DISCUSSION

Replication-defective recombinant adenoviruses have been widely studied *in vitro* and *in vivo* as a vector to deliver cancer therapeutic genes. Adenoviral-based cancer gene therapy still remains an unrealized potential for its ability to infect and transduce a variety of mammalian cells, including prostate cells [20], in a cell cycle replication-independent manner without genotoxicity. However, there are several limitations to the use of these vectors for cancer gene therapy. To overcome the defects of replication-deficient recombinant adenoviruses tissue-restricted replication competent adenoviruses have been developed. The main strategy is to control the expression of adenovirus E1a genes via a tissue-specific promoter. This strategy was used to restrict adenovirus replication in hepatocellular and prostate carcinomas via alpha-fetoprotein and PSA promoters [21, 33].

Gene therapy with *HSV-TK* as a suicide gene has been performed in a variety of tumor models *in vitro* as well as *in vivo*. Recently, Freytag et al. described a replication competent adenovirus with a suicide gene and oncolytic viral therapy with radiotherapy. They demonstrated that the suicide genes CD and HSV-TK could augment the anti-tumor effects of replication competent adenoviruses and sensitize tumor cells to ionizing radiation [23, 34-38]. However, Freytag's virus cannot be used to treat metastatic disease and its tumor-specific replication is questionable. We previously showed that both *PSA* and *OC* promoters can transcriptionally regulate *HSV-TK* gene-based therapy to inhibit the growth of AI PSA-producing cells. A PSA-selective replication-competent adenovirus, CG7870 which is similar to CV706 except that both the E1A and E1B genes are under the transcriptional control of prostate-specific promoters, was administrated intravenously to patients with hormone-refractory metastatic prostate cancer [39-41].

The current investigations build on the ability of the adenovirus to infect prostate cancer cells and provide both expanded infection and longer exogenous gene expression with a prostate-restricted replication-competent oncolytic virus, AdIU1. In previous investigations, the prostate-specific enhanced sequence (*PSES*) was developed by locating the minimal sequence, AREc3 and PSME (del2) in AREc and PSME, respectively and placing AREc3 upstream from PSME (del2) [30]. PSES showed high activity specifically in PSA/PSMA-positive and AI prostate cancer cells [31]. L2 and L5 in AREc3 did not affect transcriptional activity and was deleted and replaced 90 bp proximal region of PSME was replaced by a simple AP-3 binding site. These manipulations reduced the size of PSES from 513 bp to 407 bp. The simplified *PSES* is called m6. The tissue-specific activity of m6 has been tested in several cell lines by luciferase assay showing that m6 retains strong prostate specificity being active only in PSA/PSMA positive prostate cancer LNCaP and C 4-2 cells.

In this study, we investigated the gene-directed enzyme/pro-drug therapeutic effect of AdIU1, a novel PRRA expressing the *m6* promoter-driven *HSV-TK* suicide gene. AdIU1 can replicate and kill

infected cells by viral lysis, leading to *in vivo* amplification of input viral dose, spreading to adjacent cancer cells after lysis of initially infected cells. Additionally, AdIU1-infected cells produce HSV-TK to enhance killing by pro-drug administration [42]. The *in vitro* tissue-specific cytotoxicity of AdIU1/GCV (10 μg/ml) in CWR22rv, C 4-2 and DU-145 cells was assessed. While the growth of CWR22rv and C 4-2 cell lines was significantly inhibited by a small number of AdIU1 virus particles and GCV, the growth of the DU-145 could only be inhibited by a much greater exposure to AdIU1/GCV. This was expected, since DU-145 cells are PSA/PSMA-negative prostate cancer cells. Collectively these results demonstrate that AdIU1/GCV has selective cytotoxicity in AI, PSA/PSMA-expressing cells with a good therapeutic window. As expected, intratumoral injection of AdIU1 and treatment with GCV effectively induced growth delay of CWR22rv tumors in nude mice. H&E staining revealed a large number of fibroblasts infiltrating the virus-plus-pro-drug treated tumors. Furthermore, AdIU1/GCV-treated tumors were significantly inhibited in growth. Virus persistency was detected in AdIU1, AdE4PSESE1a, and AdE4PSESE1a/GCV-treated tumors, but not in AdIU1/GCV-treated tumors. This observation is consistent with the reporte by Freytag et al. that TK/GCV treatment inhibits adenoviral replication. The question is why AdIU1/GCV-treated tumors did not regrow after treatment was stopped. It is likely that the mass fibrosis observed in the AdIU1/GCV-treated tumors limit their growth. The other question that needs to be answered in the future is how AdIU1/GCV treatment induces mass fibrosis.

In conclusion, we have developed a suicide gene-TK armed prostate-restricted replicative adenovirus, AdIU1. AdIU1 demonstrated tumor-specific killing activity, and its tumor-killing activity could be enhanced by a prodrug, GCV. Gene therapy as monotherapy against prostate cancer currently remains in its infancy. Although preventive strategies are being entertained, the ultimate clinical use of gene therapy for improving prostate cancer treatment would most likely be in combination with surgery, radiation or chemotherapy. The next step is a combined approach to prostate cancer including enzyme pro-drug suicide gene therapy such as AdIU1 with GCV and radiation therapy or cytokine therapy to improving conventional prostate cancer treatment represent the most promising immediate clinical application for prostate gene therapy.

MATERIALS AND METHODS

Cells and Cell Culture

HER 911E4 cells, derived from adenoviral *E1* (bp 79 to 5,789)-immortalized HER 911 (human embryonic retinoblastoma) cells, express adenoviral E4 proteins under control of the *tetR* promoter. HER 911E4 cells were cultured in DMEM supplemented with 10 % FBS, 1 % penicillin/streptomycin (P/S), 0.1 mg/mL hygromycin B (Calbiochem, San Diego, CA) and 2 μg/mL doxycycline (Sigma, St. Louis, MO). AI, androgen receptor (AR) and PSA/PSMA-positive prostate cancer cell lines C4-2 and CWR22rv, and AI, AR and PSA-negative cell lines DU-145 and PC3 were cultured in RPMI 1645 supplemented with 10 % FBS and 1 % P/S. Human kidney cancer cell line RCC29, human testicular cancer cell line Tera-1, and human colon cancer cell line HT-29 were maintained in DMEM supplemented with 10 % FBS and 1 % P/S. The breast cancer cell line MCF 7 was cultured in MEM with 10 % FBS and 1 % P/S with 1 mM sodium pyruvate. HER 293 cells were cultured in MEM with 10 % FBS and 1 % P/S and 0.1 mM nonessential amino acids. The cells were maintained at 37°C in a 5 % CO_2 incubator.

Luciferase assay

The Tissue specific activity of m6 was investigated by transient transfection for luciferase assay. 2 x 10^5 cells/well were plated for 24 hours. Plasmid DNA, pGL3/m6/TATA and pGL3/TATA were delivered into the cells with DOTAP (Roche, Indianapolis, IN) following the manufacturer's protocol. 0.5-1 μg DNA was mixed with lipid at room temperature before addition to a well containing 1 mL of serum-free and phenol red-free RPMI 1640 medium. After 15 minutes, DNA-lipid complexes were added to the well and incubated for 5 hours at 5 % CO_2 and 37^{o}C. DNA-lipid containing medium was then replaced with 1 mL culture medium. After 2 days, cells were collected and lysed in 250 μL passive lysis buffer (Promega, Madison, WI). Cell lysates were vortexed for a few seconds and spun for 3 min. 10 μL of supernatant was mixed with 50 μL of luciferase substrate (Promega, Madison, WI) and measured with a femtometer (Zylux, Germany). The luciferase activity was determined by being divided by the basal activity represented by transfection of pGL3/TATA.

Construction of the prostate-restricted replicative adenovirus (PRRA) AdIU1.

The construction of the backbone for AdE4PSESE4 was described previously [31]. To construct AdIU1, the *CMV-EGFP* expression cassette in AdE4PSESE1a was replaced by a *m6-HSV-TK* expression cassette. *HSV-TK* and *E1b* in the left arm was under the transcriptional control of *m6* and *E4* and *E1a* in the right arm was controlled by PSES promoter. Figure 1 illustrates the structure of each virus used in this study. The adenoviral genome was released from the cloning vector by digestion with Pac I restriction

enzyme and transfected into HER 911E4 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). The plate was incubated at 37°C under 5 % CO_2 for 7 to 10 days after transfection until a cytopathic effect was observed. AdIU1 was further amplified in HER 911E4 cells. The recombinant adenoviruses were purified by CsCl gradient centrifugation. All gradient-purified viral stocks were dialyzed in a dialysis buffer (1 mM $MgCl_2$, 10 mM Triz HCl (pH 7.5) and 10 % glycerol) for 24 hrs at 4°C, with three buffer changes. Aliquots of purified virus were stored at -70°C. The viral titer was determined by Adeno-XTM Rapid Titer system (BD Biosciences, Palo Alto, CA) following the manufacturer's protocol. Briefly, a dilution of the viral stock in question was used to infect HER 911 E4 cells. 48 hrs later, these cells were fixed and stained with antibody specific to the adenovirus hexon protein. Signal was detected after a secondary antibody conjugated with horseradish peroxidase (HRP) amplified the signal of the anti-hexon antibody. Subsequent exposure to a metal-enhanced DAB substrate turned the infected cells dark brown. Then the titer of the stock in question could be determined by counting the number of brown cells in a given area. Each stained cell corresponded to a signal infectious unit (IFU).

Viral replication assay

CWR22rv, C4-2, PC-3 and DU-145 cells were seeded in 6-well plates (1×10^6 cells per well) 1 day prior to viral infection and subsequently infected with AdIU1 or AdE4PSESE1a (2 IFU/cell). The media were changed 24 hrs after infection, and the viral supernatants were harvested 3 days after infection. The cells were examined under light microscopy daily for up to 5 days. Then the titers of the harvested viral supernatants were determined by titer assay. HER 911E4 cells were seeded in 96-well plates (5×10^3 cells per well) 1 day prior to infection. The cells were infected with serial volume dilutions of the harvested supernatants, ranging from 1 to 10^{-11} µL per well, with each row of 8 wells receiving the same dose of virus. The media were changed on day 4, and the cells were examined under the microscope on day 7. The dose of the produced viruses was represented as an LD_{50} value, the dilution factor that caused a cytopathic effect in at least 4 wells of cells in a single row on a 96-well plate by day 7.

Western blot analyses for E1a and HSV-TK expression

To detect tissue specific expression of Ad5E1a and HSV-TK, 1×10^6 cells in 60 mm dishes were infected with AdIU1. Each cell line was infected with standardized doses of virus [31]. Cells were harvested and lysed in 100 µL of cell lysis buffer (50 mM Tris-HCl (pH 7.4), 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 µg/mL Aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM Na_3VO_4, and 1 mM NaF) 48 hrs post viral infection. Lysates were centrifuged at 14000 rpm for 20 min. and the supernatants were collected. Protein concentration was estimated by dye binding assay (Bio-

Rad, Hercules, CA). Protein (25 µg) was loaded onto a 10 % SDS-PAGE gel. The proteins were transferred to polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, CA) and the membrane was probed with antibodies reactive to Ad5E1a protein (BD Bioscience) or TK polyclonal serum (provided by M. Black, Department of Pharmaceutical Sciences, Washington State University, Pullman, WA). and visualized by an enhanced chemiluminescence kit (Amersham Life Science, Piscataway, NJ).

Dose-dependent *in vitro* cell killing assay

CWR22rv and DU145 cells were seeded onto 24-well plates at a density of 1.5×10^5 or 1×10^4 cells/well respectively. After 24 hrs, the cells were infected with 0.0002-2 IFU per cell of AdIU1 or AdE4PSESE1a. 24 hrs after infection, the media were removed and replaced by fresh media with or without GCV (10 µg/ml). Media with or without GCV were changed every 2 days. Viable cells were analyzed by crystal violet assay 7 days post-infection.

Time-dependent *in vitro* killing assay

CWR22rv and DU-145 cells were plated in 24-well plates. Cells were divided into 4 treatment groups, no treatment, AdIU1 (0.2 IFU/cell), GCV, and AdIU1 (0.2 IFU/cell) plus GCV. The media were changed 24 hrs after infection, and GCV (10 µg/ml) was added 24 hrs after the media change. Cell viability was analyzed at day 1, 3, 5 and 7 by crystal violet assay.

***In vivo* evaluation of AdIU1 therapy**

All animal methods and procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC). CWR22rv xenografts were established by injecting 2×10^6 cells s.c in the flanks of 6 week-old male, athymic nude mice. The injected mice were castrated 3 days after cell injection. Mice with similar tumor sizes (3 – 5 mm) were divided into four groups receiving, AdE4PSESE1a (control PRRA), AdE4PSESE1a plus GCV, AdIU1, or AdIU1 plus GCV treatment. 2×10^9 virus particles of either AdE4PSESE1a or AdIU1 in 100 µL PBS were injected intratumorally. 5 days after virus injection, GCV (40 mg/kg body weight) was administered i.p. twice daily for 10 days. Tumor sizes were measured every 5 days, and the following formula was applied to calculate tumor volume length x width^2 x 0.5236. Mice were sacrificed and tumors harvested for histological examination 30 days after injection.

Histology and Immunohistochemistry

Tumors were harvested, immediately fixed in formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (H&E) according to the standard protocol. For

immunohistochemistry, tumor sections were deparaffinized, rehydrated and heated in a microwave oven for 20 min in activity antigen-retrieval solution (10 mM citric buffer, pH 6.0). Endogenous peroxidase was inactivated with 3% hydrogen peroxide solution. The slides were rinsed with distilled water, washed twice with PBS for 3 min and incubated with Superblock (Scytek Laboratories, Burlingame, CA) in a humidified chamber for 1 hr at room temperature. After rinsing with PBS, the slides were incubated with avidin (Vector Laboratories, Inc., Burlingame, CA) for 15 min, washed with PBS and blocked with biotin in a humidified chamber for 15 min at room temperature. A monoclonal mouse antibody to adenovirus type 5 (Abcam, Cambridge, MA) was applied. The slides were incubated with primary antibodies overnight in humidified chambers at 4 °C. After PBS rinse, a biotinylated secondary antibody was applied to the slides and incubated for 1 hr. After washing with PBS, slides were incubated with avidin-peroxidase complex (ABC) reagent (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min, washed once with PBS, stained with freshly prepared diaminobenzidine (DAB) solution for 15 min and counterstained with hematoxylin.

***In situ* Terminal Deoxynucleotide Transferase-Mediated Nick End Labeling Assay**.

The *in situ* apoptosis detection kit was purchased from Roche Diagnostics. Tumor tissue sections were deparafinized using a sequential xylene protocol and rehydrated through gradients of ethanol and distilled water. Slides were treated with 10 nmol/L Tris solution containing 1 µg/ml proteinase K for 15 min. All slides were rinsed with PBS and incubated with 100 µL terminal deoxynucleotidyl transfererase-mediated nick end labeling (TUNEL) reaction mixture (or 100 µL control labeling solution for negative control) in a humid chamber at 37^{o}C for 30 min. The slides were washed 3 times with PBS and incubated with 100 µL TUNEL POD solution in a humid chamber at 37^{o}C for 30 min. After washing with PBS, the slides were stained with freshly prepared DAB solution for 10 min, rinsed with PBS, and counterstained with hematoxylin.

Figure Legend

FIG. 1. A. Sequences of the enhancer core of the PSA gene, AREc3, located in the 4.3 kb upstream of PSA promoter. Sequence analysis of AREc3 revealed 6 putative GATA transcriptional factor binding sites besides a reported 3 androgen response elements. **B.** Sequences of PSME located in the third intron in the PSMA gene (*FOLH*1). PSME is characterized by the repeat sequence (marked by underline and bold). Several potential transcription factor binding sites, such as AP-1, AP-3, and SRY/SOX, are indicated. **C.** pGL3/m6/TATA (0.5 μg) was transfected into various cell lines (2 x 10^5 cells for each). After 2 days, cells were harvested, lysed with passive lysis buffer and analyzed for luciferase activity. This experiment was conducted in the absence of androgen. Luciferase activity was determined by being divided by the basal activity represented by transfection of pGL3/TATA. pGL3/m6/TATA is active only in PSA/PSMA positive LNCaP and C4-2 cells.

FIG. 2. Schematic illustration of AdIU1. AdIU1 was constructed by placing adenoviral *E1a* and *E4* genes under the control of *PSES* to direct adenovirus replication, and *HSV-TK* gene, a pro-drug enzyme gene, under the control of *m6* enhancer to maximize cell-killing activity through a bystander effect.

FIG. 3. Expression of Ad5 E1a and HSV1-TK proteins by AdIU1 was evaluated in different cell lines. CWR22rv, C 4-2, PC 3 and DU-145 were infected with AdIU1. 48 hrs post viral infection, cell lysates were collected and Western blot was performed. A large amount of E1a proteins ranging in size from approximately 35-46 kDa and HSV-TK (40-50 kDa) were detected in AdIU1- infected CWR22rv and C 4-2 cells. AdIU1- infected DU-145 and PC 3 did not express a detectable amount of E1a and HSV-TK proteins.

FIG. 4. Dose or time-dependent *in vitro* killing assay. 1.5 X 10^5 CWR22rv and DU-145 cells were seeded in 24-well plates, infected by serial dilutions of AdIU1 from 0.0002 to 2 IFU/cell, with replicative-deficient adenovirus AdE1aPSESE4 as a controls, and then treated with or without GCV (10 μg/mL). Seven days after infection, cells were stained with crystal violet (A). CWR 22rv and DU-145 were treated with 0.2 IFU/cell of AdIU1 (■), 10 μg/mL of GCV (▲) or AdIU1 plus GCV (●). A group of cell without treatment (♦) were used as a control. At days 1, 3, 5, and 7, crystal violet staining was performed to detect attached cells. Then 1% SDS was added to lyses the cells and for OD_{590} reading. Cell

survival rate curves were drawn to evaluate the killing activity of AdIU1. The growth of AdIU1-Infected cells was significantly inhibited by the addition of Ganciclovir (GCV) (10 µg/mL), especially in CWR22rv (B). On the other hand, the growth of DU-145 (C) was not inhibited by AdIU1 plus GCV treatment.

FIG. 5. Tumor growth inhibition by AdIU1 plus GCV in a xenograft prostate model. CWR22rv prostate tumor xenografts were established s.c. in athymic nude mice. Tumors were treated with AdE4PSESE1a (♦, n=7), AdE4PSESE1a plus GCV (■, n=8), AdIU1 (▲, n=8) or AdIU1 plus GCV (●, n=8). Viruses were delivered by intratumoral injection at day 0 and GCV (80 mg/kg of body weight/day) was administered 2 times a day for 10 days. Tumor volumes were measured every 5 days. Treatment with AdIU1 plus GCV significantly inhibited the growth of CWR22rv tumors compared to treatment of the AdIU1group.

FIG. 6. Histologic representations of virus treated tumors. Tumor sections of AdIU1 plus GCV (A), AdIU1 (B), AdE4PSESE1a plus GCV (C) and AdE4PSESE1a (D) treatment groups were stained with hematoxylin and eosin (H&E). The lower left panel of each picture was taken at low power (4X magnification), and the upper right panel is magnified to 40 x (focused on white box). Tumor treated with AdIU1 plus GCV (A) demonstrated marked fibrosis and infiltration of fibroblasts (arrows). AdE4PSESE1a and plus GCV and AdIU1 alone treated tumors revealed large areas of necrosis embedded within healthy tumor foci.

FIG. 7. Apotosis signal and Ad5E1a expression in subcutaneous tumors. All necrosis spots showed apoptotic signals by TUNEL Assay (a, AdIU1 plus GCV, b, AdIU1, c, AdE4PSESE1a Plus GCV, d, AdE4PSESE1a). In the AdIU1 with GCV group, E1a expression was rare (e, AdIU1 Puls GCV, f, AdIU1, g, AdE4PSESE1a Plus GCV, h, AdE4PSESE1a).

Table Legend.

TABLE 1: Tissue/tumor-specific replication ability of AdIU1

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REFERENCES

1. Jemal, A., et al. (2006). Cancer statistics, 2006. *CA Cancer J Clin* 56: 106-130.
2. Cheon, J., Kim, H. K., Moon, D. G., Yoon, D. K., Cho, J. H., and Koh, S. K. (2000). Adenovirus-mediated suicide-gene therapy using the herpes simplex virus thymidine kinase gene in cell and animal models of human prostate cancer: changes in tumour cell proliferative activity. *BJU Int* 85: 759-766.
3. van der Poel, H. G., et al. (2001). A novel method for the determination of basal gene expression of tissue-specific promoters: an analysis of prostate-specific promoters. *Cancer Gene Ther* 8: 927-935.
4. Latham, J. P., Searle, P. F., Mautner, V., and James, N. D. (2000). Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res* 60: 334-341.
5. Steiner, M. S., and Gingrich, J. R. (2000). Gene therapy for prostate cancer: where are we now? *J Urol* 164: 1121-1136.
6. O'Keefe, D. S., et al. (2000). Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate* 45: 149-157.
7. Xing, Y., et al. (2002). [Bystander effect mediated by herpes simplex virus-thymidine kinase/ganciclovir approach on prostatic cancer cells and its regulation]. *Zhonghua Yi Xue Za Zhi* 82: 1484-1487.
8. Park, H. S., et al. (2003). In vivo characterization of a prostate-specific antigen promoter-based suicide gene therapy for the treatment of benign prostatic hyperplasia. *Gene Ther* 10: 1129-1134.
9. Ko, S. C., et al. (1996). Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res* 56: 4614-4619.
10. Springer, C. J., and Niculescu-Duvaz, I. (2000). Prodrug-activating systems in suicide gene therapy. *J Clin Invest* 105: 1161-1167.
11. Denny, W. A. (2003). Prodrugs for Gene-Directed Enzyme-Prodrug Therapy (Suicide Gene Therapy). *J Biomed Biotechnol* 2003: 48-70.
12. Schepelmann, S., and Springer, C. J. (2006). Viral vectors for gene-directed enzyme prodrug therapy. *Curr Gene Ther* 6: 647-670.
13. Yu, D. C., Sakamoto, G. T., and Henderson, D. R. (1999). Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res* 59: 1498-1504.
14. Matsubara, S., et al. (2001). A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res* 61: 6012-6019.
15. DeWeese, T. L., et al. (2001). A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res* 61: 7464-7472.
16. Sadeghi, H., and Hitt, M. M. (2005). Transcriptionally targeted adenovirus vectors. *Curr Gene Ther* 5: 411-427.
17. Wu, L., et al. (2001). Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Ther* 8: 1416-1426.
18. Diamandis, E. P., and Yousef, G. M. (2002). Human tissue kallikreins: a family of new cancer biomarkers. *Clin Chem* 48: 1198-1205.
19. Hsieh, C. L., Gardner, T. A., Miao, L., Balian, G., and Chung, L. W. (2004). Cotargeting tumor and stroma in a novel chimeric tumor model involving the growth of both human prostate cancer and bone stromal cells. *Cancer Gene Ther* 11: 148-155.
20. Kubo, H., et al. (2003). Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther* 14: 227-241.
21. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* 57: 2559-2563.

22. Galanis, E., Vile, R., and Russell, S. J. (2001). Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors. *Crit Rev Oncol Hematol* 38: 177-192.
23. Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D., and Kim, J. H. (1998). A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther* 9: 1323-1333.
24. Freytag, S. O., et al. (2002). Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res* 62: 4968-4976.
25. Freytag, S. O., et al. (2003). Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res* 63: 7497-7506.
26. Edwards, S. J., et al. (2002). Evidence that replication of the antitumor adenovirus ONYX-015 is not controlled by the p53 and p14(ARF) tumor suppressor genes. *J Virol* 76: 12483-12490.
27. Rothmann, T., Hengstermann, A., Whitaker, N. J., Scheffner, M., and zur Hausen, H. (1998). Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J Virol* 72: 9470-9478.
28. O'Shea, C. C., et al. (2004). Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell* 6: 611-623.
29. O'Shea, C. C., Soria, C., Bagus, B., and McCormick, F. (2005). Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. *Cancer Cell* 8: 61-74.
30. Lee, S. J., et al. (2002). Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol Ther* 6: 415-421.
31. Li, X., et al. (2005). Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer. *Cancer Res* 65: 1941-1951.
32. Lee, S. J., et al. (2003). NFATc1 with AP-3 site binding specificity mediates gene expression of prostate-specific-membrane-antigen. *J Mol Biol* 330: 749-760.
33. Hallenbeck, P. L., et al. (1999). A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther* 10: 1721-1733.
34. Kim, J. H., Kim, S. H., Brown, S. L., and Freytag, S. O. (1994). Selective enhancement by an antiviral agent of the radiation-induced cell killing of human glioma cells transduced with HSV-tk gene. *Cancer Res* 54: 6053-6056.
35. Kim, J. H., Kim, S. H., Kolozsvary, A., Brown, S. L., Kim, O. B., and Freytag, S. O. (1995). Selective enhancement of radiation response of herpes simplex virus thymidine kinase transduced 9L gliosarcoma cells in vitro and in vivo by antiviral agents. *Int J Radiat Oncol Biol Phys* 33: 861-868.
36. Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H., and Freytag, S. O. (2000). Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. *Hum Gene Ther* 11: 67-76.
37. Rogulski, K. R., et al. (2000). In vivo antitumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy. *Cancer Res* 60: 1193-1196.
38. Freytag, S. O., et al. (2002). Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. *Int J Radiat Oncol Biol Phys* 54: 873-885.
39. Small, E. J., et al. (2006). A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol Ther* 14: 107-117.
40. Dilley, J., et al. (2005). Oncolytic adenovirus CG7870 in combination with radiation demonstrates synergistic enhancements of antitumor efficacy without loss of specificity. *Cancer Gene Ther* 12: 715-722.
41. Chen, Y., et al. (2001). CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity. *Cancer Res* 61: 5453-5460.

42. Wang, J. Q., Zheng, Q. H., Fei, X., Mock, B. H., and Hutchins, G. D. (2003). Novel radiosynthesis of PET HSV-tk gene reporter probes [18F]FHPG and [18F]FHBG employing dual Sep-Pak SPE techniques. *Bioorg Med Chem Lett* 13: 3933-3938.